ISOLATED BONE CELL TYPES: FUNCTIONAL CHARACTERIZATION AND PTH-INDUCED IN VITRO DIFFERENTIATION

Jay Arthur Jensen

1980

Isolated Bone Cell Types: Functional Characterization and PTH-induced *in vitro* Differentiation

Jay Arthur Jensen

AB Harvard College, 1976

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Medicine

Yale University School of Medicine

1980

for my mother and father

Table of Contents

And he said unto me, Son of man, can these bones live?
And I answered, O Lord God, thou knowest.

Ezekiel 37:3

Bone has long defied those who sought to understand it. As religious relic and pirate symbol, bone was representative to previous generations of both life and death. Scientific efforts to determine if "these bones live" have only lent support to the ancient notion that, in bone, life is somehow trapped within a surrounding shell: living cells engulfed by calcified matrix.

That bone cells have different functions was first postulated by Kollicker in 1873 on the basis of morphologic evidence. He described osteoclasts-- multinucleate giant cells-- believing them to be the active agents of bone resorption. Several other bone cell types have since been described, including osteoblasts, osteocytes, and osteoprogenitor cells. Similarly, the functions of bone have been more expansively characterized. Not only does bone provide protection and mechanical support, but also is integrally involved in mineral homeostasis. Efforts to elucidate how each bone cell type contributes to the various functions of bone has required a variety of experimental approaches. Bone has been studied in systems involving whole animals, as well as in tissue culture. A relatively new approach to the study of bone cells has involved their isolation from the collagen matrix. The validity of this approach is dependent upon the biochemical and functional characterization of the isolated bone cells.

Isolated bone cells are metabolically active. In culture, the bone cells have been shown to synthesize collagen, resorb bone matrix, and respond to hormones at near physiological concentrations. Although the cells retain the ability to perform some differentiated functions in culture, their retention of the appearance of differentiated cells has been linked to culture surface.

The separation of the isolated bone cells into distinct populations of osteoblasts and osteoclasts has constituted a special challenge. Hormonal, ionic, and other pharmacologic or physiologic stimuli may have opposing effects on osteoblasts and osteoclasts. Therefore, the effects of these stimuli on separated populations of cells would add to the understanding of cell function in intact bone.

Isolated bone cells

In 1964, Peck and coworkers(1) reported the dispersion of viable cells from rat calvaria following short term incubation in buffered crude collagenase. Frontal and parietal bones were dissected from 17-21 day rat fetuses and incubated for 90 minutes. Histological studies of the collagenase treated bone showed no areas were spared; and, thus, there was no selective removal of cell types. The viability of the released cells was assessed by vital dye uptake and multiplication in tissue culture. The isolated cells were further characterized by measurement of glucose metabolism. Freshly harvested cells were similar to whole bone

segments in aerobic glucose metabolism. Of particular interest was a morphologic change observed in the cells after subculturing. Round and stellate cells were noted in primary cultures of the cells, while all cells assumed a stellate shape after subculturing. Peck showed the cells to be viable but could not prevent de-differentiation of cells on the siliconized surface of the culture vial or overgrowth of the culture with contaminating fibroblasts.

Modifications of the method employed by Peck have been developed. Dziak and Brand(2) showed that fragments of calcified matrix can contaminate the bone cell mixture. This contamination was responsible for erroneously high values for total cell calcium which proved to be significant in studies of calcium transport. Whether contamination of preparations of isolated bone cells with fragments of their calcified matrix would lead to other inaccuracies is unknown. The contamination was removed by filtration of the cell suspension through a 35μ mesh nylon net and a five minute treatment with a cold, pH 6, isotonic salt solution. The viability of the cells isolated by this procedure was evaluated using trypan blue dye exclusion. This method showed 90-95% of the cells excluded dye. The question of whether the 35μ mesh nylon net filtered out osteoclasts larger than 35μ was not addressed.

Functional characteristics of isolated bone cells

To accept isolated bone cell preparations as a valid *in vitro* experimental representation of cells in bone requires the

demonstration that the isolated cells are capable of performing functions generally attributed to bone cells. In skeletal remodeling, for example, bone cell types function in a coordinated manner to replace old bone with new bone.

The sequence of events at the remodeling site has been studied(3). Preosteoclasts are activated to fuse into a multinucleated osteoclast. The osteoclast resorbs the bone, forming Howship's lacunae. Then, after a time lag, osteoblasts appear in the same area and form new bone. The remodeling sequence is therefore Activation-Resorption-Formation and includes the basic functions expected of isolated bone cells.

Activation

The signal which activates mesenchymal and/or blood borne cells to become osteoclasts is unknown. Local factors may include piezo-electricity(4) or osteoclast activating factor(5,6). A prostaglandin (E_2) has been shown to stimulate bone resorption in tissue culture(7) as have active vitamin D metabolites(8,9). The stimulatory effects of parathyroid hormone on bone resorption have long been recognized(10), and it is known that the rate of activation is increased by this hormone (infra vide).

The mechanism of bone cell activation has been difficult to study partially because of the difficulty in using preparations of bone:

> The presence of a calcified extracellular matrix is a severe handicap to the analysis of ionic and metabolic events within bone cells. In addition, because of the apparently unique extracellular environments of all bone cells, the heterogeneity

of bone cell types and functions, and the difficulty or impossibility of obtaining bone intact in the functional sense, metabolic studies on isolated bone fragments and bone cells are difficult to perform and interpret.(10)

Isolated bone cells may provide a system in which the effect of postulated osteoclast activators on mesenchymal osteoprogenitor cells might be studied. For instance, in a calcified matrix, bone cells have been found only to respond to vastly supraphysiologic concentrations of PTH. Using isolated cells, PTH has been shown to stimulate increases in cyclic AMP levels at physiologic concentrations(11).

Evidence has accumulated that while osteoblasts arise from local progenitor cells, osteoclasts arise from blood-borne precursors. Gothlin and Ericsson(12) studied fracture healing with autoradiography in parabiotic rats. The rats were parabiosed, irradiated (with hindlimbs of one rat shielded), the right femur fractured in both animals, and tritiated thymidine administered to the protected rat (after cross circulation had been arrested). At intervals, the rats were re-parabiosed. Autoradiographic studies showed that osteoblasts, chondroblasts, fibroblasts, and osteoclasts were labeled in the regeneration blastema of the femur in the protected rat. Only osteoclasts were labeled in the unprotected rat. The authors concluded that osteoclasts must have arisen from blood-borne cells. In similar experiments, Buring(13) found that only macrophages and osteoclasts were labeled in the unprotected rat.

Resorption

Resorption in bone is mediated by osteoclasts. The zone in which osteoclasts make contact with the bone is characterized by the presence of a ruffled border and dense basophilic patches on each side of it, called the sealing zone. The lysozomal enzymes contained in the cytoplasm of the cell are released(14) into the enclosed space between the osteoclast and the bone. In addition to the acid phosphatases, the osteoclast produces citric, carbonic, and lactic acids, reducing the pH in the resorption area. This reduction in pH provides for optimal lysozomal enzyme activity and for the dissolution of the hydroxyapatite crystals. The cells may also produce a collagenase to aid in the digestion of the bone matrix.

Isolated bone cells have been examined for enzymes thought to participate in the process of bone resorption. Acid phosphatase, known to be present at greater concentrations in osteoclasts(43), is present in isolated bone cells(37). A collagenase which had been previously described in bone culture fluids and extracts of whole bone(15), is secreted by isolated bone cells(16). Furthermore, PTH stimulates collagenase secretion by the isolated cells(17).

A method to assay the functional resorptive activity of isolated bone cells evolved from a resorption assay developed from work in tissue culture. While investigating factors influencing the response of bone to PTH, Raisz(18), developed a sensitive bioassay for bone resorption. Resorption was measured by release

of radioactive calcium previously incorporated into embryonic bone. The bone was prepared by injecting pregnant rats on the 18th day of gestation with ^{45}Ca or ^{3}H-proline. After 24 hours, bones were removed, dissected free of soft tissues, and transferred to a culture vial.

The adaptation of this assay for isolated cells(19) involved killing the bone after dissection from soft tissues. After bone cell death was certain in the prelabeled bone (accomplished by repeated freeze-thawings and exposure to ultraviolet light for several hours) the bone was cultured with or without isolated cells prepared from other bone samples.

Bone cells isolated from fetal rat calvaria cause matrix resorption and mineral release when cultured on devitalized bones(19). However, other cell types, including fibroblasts and spleen cells, tested with this assay showed no ability to release mineral or matrix.

The ability of PTH to stimulate bone resorption is recognized in vivo and in tissue culture. The response to PTH is an increase in the number of osteoclasts(20) and an increase in the activity of osteoclasts(21). In tissue culture, increasing the phosphate concentration over the range of 0.25 to 4.0 mM produced a decreased release of calcium from bone, particularly in response to PTH(22). Calcium concentration can be varied from 5-12 mg per 100 ml and pH from 6.8 to 7.4 without inhibiting the resorptive response to PTH(23).

Formation

The bone matrix is formed by collagen fibers and ground substance. Spindle shaped crystals of hydroxyapatite are found on and in the collagen fibers and in the ground substance. The ground substance is composed essentially of carbohydrate protein complexes: glycosaminoglycans, with a predominance of chondroitin sulfate and small amounts of hyaluronic acid and glycoproteins, mainly sialoprotein.

Several methods have been developed to measure collagen synthesis. The most common method assays the conversion of proline to hydroxyproline. Because hydroxyproline has been found in virtually no other mammalian protein, its formation permits its use in identifying collagen.

The claim that collagen formation can be assayed as hydroxyproline production had its basis in the work of Stetton and Schoenheimer(24). Working during the 1940's these investigators fed ^{15}N-proline to rats. They found that proline and hydroxyproline equally labeled in the rats' collagen. Later, when Stetton prepared labeled ^{15}N-hydroxyproline and fed it to rats, essentially none of the label was found to be incorporated into collagen. Further investigation revealed that a protein intermediate, now called protocollagen, contained more proline than mature collagen. This is the initial synthetic product in which some of the proline is subsequently converted to hydroxyproline. The enzyme carrying out this conversion has been identified as protocollagen hydroxylase.

There are several cofactors and cosubstrates required for hydroxylation of proline to hydroxyproline in protocollagen. Molecular oxygen, alpha-ketoglutarate, and a reducing agent such as ascorbic acid are all required. Because iron is an essential cofactor of the enzyme, metal chelators such as alpha, alpha'-dipyridyl inhibit the hydroxylation of proline.

Other methods proposed to assay collagen measure the in vitro activity of protocollagen hydroxylase(25,26). The enzyme is retained from the supernatant following the lysis and centrifugation of a cell monolayer. It must be noted that these methods do not assay collagen formation, but rather the activity of the enzyme known to hydroxylate residues of a known collagen precursor.

Methods assaying collagenase digestible protein(27) are dependent on the purity of the collagenase used(28).

Isolated bone cells were found to synthesize collagen in cell culture(29). Collagen synthesis was determined by the incorporation of radioactive proline into the peptide hydroxyproline. Furthermore, using isolated bone cells, ascorbic acid was shown to promote collagen synthesis by directly stimulating the hydroxylation of a proline rich peptide(30).

Collagen synthesis is affected by PTH and the concentration of inorganic orthophosphate in the medium. In tissue culture, PTH was found to decrease the incorporation of proline in collagen digestible protein(27). Furthermore, this effect was specific for bone collagen, as the incorporation of collagen digestible protein was not inhibited in cartilage collagen. The effect

of inorganic orthophosphate concentration on rates of collagen formation has also been studied in tissue culture(31). The rate of collagen synthesis was markedly reduced when the phosphate concentration was lowered to 0.32 mM. Increasing the phosphate concentration beyond 1.30 mM did not increase the rate of collagen formation.

Differentiation of isolated bone cells

In the first description of bone cells isolated by enzymatic digestion, Peck et al. observed that while round and stellate cells were noted in primary cultures, all cells assumed a stellate appearance after subculturing. Dedifferentiation of the cells or overgrowth of fibroblasts could not be ruled out. Miller et al.(32) noted morphologic transformation from spherical to stellate shape accompanied cell maturation in culture. Not only could the transformation be seen spontaneously, but could also be induced by removal of fetal calf serum from the media, exposure to PTH, epinephrine, or cyclic nucleotide analogues.

Evidence most suggestive of cell dedifferentiation following multiple subculturings is biochemical rather than morphologic. Peck et al.(11) observed that repeated subculturing decreases the sensitivity of bone cells to PTH as measured by cyclic AMP production. This phenomena suggests a loss of number and/or affinity of cellular receptors for PTH, or of adenylate cyclase molecules. Alternatively, a preferential overgrowth of cells which were initially PTH insensitive may occur with subculturing.

Altman et al.(33) cultured fetal rat calvarial cells on the endosteal surface of devitalized bone. In contrast to cells cultured in petri dishes without bone, the cells cultured on the bone surface differentiated into cells which resembled the heterogenous collection of cells found in normal bone. Some of the cultured calvarial cells were noted to resemble multinucleated osteoclasts. Cells cultured on petri dishes without bone maintained a fibroblastoid appearance throughout the culture period. No multinucleated forms were found in cell cultures maintained for periods up to 28 days.

There would seem to be no question that the surface characteristics of a cell culture influence the morphologic appearance of the cells. It is worth noting, however, that despite Altman's claim, no evidence exists that cells differentiate in response to culture surface. The cells used were never shown to have dedifferentiated.

Separation of isolated bone cell types

A limitation of preparations of isolated bone cells is the heterogenous nature of the cells. Osteoclasts have functions different than osteoblasts, and would be expected to respond to stimuli in a different, if not opposite, way. The metabolic control of the cells could be better studied if more homogenous subpopulations of bone cells could be isolated.

Several methods for separating specific bone cell types have been described. All methods start with rat or fetal rat bone

and involve manual dissection and/or sequential digestion of the tissue by bacterial collagenase. Cells are thus separated according to anatomic position or depth in the tissue(34).

Smith et al.(35) devised an entirely mechanical method of separation. Dissected segments of bone were shaken in media to disperse cells. The cells were identified solely by histologic methods. The authors presented evidence that the response to parathyroid hormone extract differed between the various subpopulations of cells. Osteoclasts were not separated by this method and no mention was made of what had become of the osteoclasts.

Yagiela and Woodbury(36) combined the method of Peck with a more rigorous dissection technique designed to lessen soft connective tissue contamination. Histologic examination of the fetal rat calvaria revealed that most of the osteoblasts were located in the central portions of the frontal and parietal bones. An excision of these areas and subsequent incubation with crude collagenase produced a cell mixture judged to be 85-90% osteoblasts.

Luben et al.(37,38,39) collected cells produced by sequential enzymatic digestion. The subpopulations differed from each other in morphology (not published) and in response to parathyroid hormone and calcitonin. The first two populations of cells removed by this procedure were labeled "CT" cells, the last three populations were labeled "PT" cells. Biochemical characterization after a primary cell culture of 7 days revealed that "CT" cells have high basal levels of acid phosphatase and hyaluronate

synthesizing capacity. In contrast, "PT" cells exhibit high levels of prolyl hydroxylase, collagen synthesis, citrate decarboxylation, and alkaline phosphatase. The "CT" cells were identified as osteoclasts, the "PT" cells as osteoblasts. PTH was found to stimulate resorption of devitalized bone by cultured "CT" cells(40).

Rather than separate cells according to anatomic position and produce populations of cells which are, at best, enriched with certain cell types, Puzas, Vignery, and Rasmussen(34) sought to utilize cell surface charge density for separation. Cell surface charge density, thought to be specific for idividual cell types(41), is exploited using free flow electrophoresis(FFE). The principles of FFE have been applied to problems of cell separation(42). An electrolyte solution flows across lines of an electric field. The sample mixture is injected continuously into the stream at a defined point. The components of the sample mixture with different electrophoretic mobilities are deflected at different angles and, hence, arrive at different ports at the bottom of the chamber. The deflection angle, designated in Figure 1, is obtained from the relation

$$\tan \alpha = \frac{\text{velocity of electrophoretic migration}}{\text{streaming velocity of buffer solution}}$$

Using the FFE technique, Puzas was able to separate three different cell populations from a heterogenous population of bone cells. When cells from a complete digestion of fetal rat calvaria were subjected to FFE, the pattern observed in Figure 2, panel A

was obtained. From an integral analysis, cells with the greatest electrophoretic mobility, peak I, represent 5% of the total, cells in peak II represent 60% of the total, and cells with the least electrophoretic mobility, peak III, represent 35% of the total. Morphological studies, done on intact fetal rat calvaria, correlated the proportions of various bone cell types in intact calvaria with the proportions of cells found in the three peaks following FFE: 5% of the cells were osteoclasts lining the resorbing areas of bone; 38% were osteoblasts and osteoprogenitor cells; and 58% were fibroblasts and loose connective tissue cells.

If cells removed from the enzymatic digestion after 20 minutes are subjected to FFE, the pattern obtained in Figure 2, panel B is obtained. Correlative morphologic evidence, histologic studies done on the calvaria after 20 minutes of digestion, permitted Puzas to identify peak II cells as fibroblasts. Luben and Wong(39) had previously shown the cells released after 20 minutes of digestion are unresponsive to PTH or calcitonin. If the cells recovered from 20-120 minutes of collagenase digestion are sujected to FFE, the pattern shown in Figure 2, panel C is obtained. The cells are resolved into two distinct peaks by FFE.

The activity/cell of alkaline and acid phosphatase from the separated cells is displayed in Figure 3. The cells in peak I contain the largest cell content of the osteoclast marker enzyme acid phosphatase. Alkaline phosphatase is distributed evenly across the cell pattern. Careful histochemical staining of bone for alkaline phosphatase(43) indicates that nearly all cell types contain this enzyme.

Acetazolamide is a specific inhibitor for carbonic anhydrase, which has been postulated to alter pH at sites of bone resorption. Using ^{3}H-acetazolamide in autoradiographic experiments, Gay et al.(44) showed that osteoclasts bound greater amounts of label than other bone cell types in bone. Utilizing this observation, Puzas incubated isolated bone cells with ^{3}H-acetazolamide before electrophoretic separation. As shown in Figure 4, the cells from peak I contain more ^{3}H-acetazolamide than cells from any other fraction.

Using these and other morphometric and biochemical studies, the authors concluded that peak I was comprised of osteoclasts and preosteoclasts, peak II of fibroblasts, and peak III of osteoblasts and preosteoblasts.

In a later experiment, Puzas(45) studied the effect of parathyroid hormone and calcitonin on cyclic AMP production in the separated bone cells. The results show peak I cells responsive to both PTH and calcitonin, peak III cells only responsive to PTH, and peak II cells not responsive to either hormone. Peak I cells, therefore, have the same hormonal responsiveness as the "CT" cells of Luben et al. which were labeled osteoclast enriched, while peak III cells have the same responsiveness as the "PT" cells, labeled osteoblast enriched.

Functional characterization of the electrophoretically separated cells is required before accepting them as specific bone cell types.

Materials and Methods

Isolation and Separation

Cells were isolated from the calvaria of 20-21 day old fetal rats using a modification of the method of Dziak and Brand(2). The calvaria were removed aseptically, and gently cleaned of adherent connective and nervous tissue. Enzymatic digestion of the calvaria was achieved in Minimum Essential Medium (Earle's #109) containing penicillin 500 units/ml, streptomycin 500 μg/ml, bovine serum albumin (fraction V) 1 mg/ml and bacterial collagenase (Worthington, type II) 1 mg/ml. Minimum Essential Medium, bovine serum albumin, penicillin and streptomycin were obtained from the Grand Island Biological Company. The calvaria were incubated at 4 calvaria/ml of incubation medium. The medium was maintained at 37° C in a water bath, shaking at 60 oscillations/minute, for 120 minutes.

Following incubation, the cell rich incubation medium was decanted from the incubation flask into a 10 ml disposable syringe fitted with a Millipore Swinnexfilter. The undigested bone fragments were washed with enzyme-free incubation medium 3-4 times, with decanting and filtration of the medium each time. The fluid containing cells was collected in centrifuge tubes (Falcon) and was resuspended in enzyme-free medium and recentrifuged two times. Resuspended in enzyme-free medium, the cells were allowed to recover from isolation for 2 hours in a slowly shaking 37° C water

bath.

The isolated cells were prepared from free flow electrophoretic separation. They were washed three times in cold separation buffer, counted, using a Levy hemacytometer, and injected into the FFE apparatus at a concentration of 20 x 10^6 cells/ml.

Free flow electrophoresis of the isolated cells was conducted using a Garching Instrumente FF-5. The sterilized separation buffer consisted of 15 mM Hepes, 10 mM glucose, 0.2 mM $CaCl_2$, 270 mM glycine, 5 mM KOH, at pH 7.24. The electrode buffer was 75 mM Hepes at pH 7.24. The field strength was 60 volts/cm; suction pump setting was 120. The separation, collection, and injection chambers were all maintained at 6° C.

Culture of bone cells

As they came from the separation chamber, cells were collected under sterile conditions in tubes containing 3 ml of culture media. The cells were collected from ports 22 through 39 as described by Puzas. They were then pooled into six groups (A-F): A(fractions 22-24), B(fractions 25-27), C(fractions 28-30), D(fractions 31-33), E(fractions 34-36), and F(fractions 37-39). The culture media consisted of Minimal Essential Media, penicillin 500 units/ml, streptomycin 500 μg/ml, 10% fetal calf serum, and, depending on the experimental design, 100 ng/ml PTH (infra vide). The cultures were incubated at 37° C. The culture media was changed at 24 hours; cultures were harvested at 48 hours. At the conclusion of cultures, pH of the culture wells was determined not to be below 7.0.

Resorption culture

Prelabeled, devitalized calvaria were prepared for cell culture by a method analogous to Mundy et al.(19). Pregnant rats at 18-19 days gestation were injected with 400μCi $^{45}CaCl_2$ (New England Nuclear) 48 hours prior to sacrifice. Fetal rat calvaria were dissected and scraped free of loose connective tissue. The calvaria were frozen and thawed three times and placed in sterile microtiter wells. After being dessicated and exposed to ultraviolet radiation for 48 hours, the calvaria were stored for use.

Isolated, separated bone cells were seeded onto the calvarial surface in 50μl volumes. Following the method of Luben et al.(40), the cells were allowed to settle for 15 minutes before an additional 950μl of culture media was added to the well. Samples of the culture media were taken at 24 and 48 hours.

To determine ^{45}Ca release from the prelabeled, devitalized calvaria in the absence of bone cells, calvaria were cultured without cells. The ^{45}Ca released from the calvaria during 24 hour intervals was compared to the total ^{45}Ca in the calvaria (infra vide). As a percentage, this baseline release of ^{45}Ca was used to adjust sample counts taken from cultures containing cells and calvaria.

At the conclusion of the incubation, total calvarial ^{45}Ca was measured. The calvaria were hydrolyzed in 6.0 N HCl at 110° C for 24 hours. The hydrolyzate was dried under N_2 at 37° C and resuspended in 1.0 ml distilled, deionized water. ^{45}Ca remaining in

the calvaria was measured. Baseline release of ^{45}Ca was deducted from sample counts based upon total calvarial ^{45}Ca content. To avoid measurement of freely exchangable calcium, only ^{45}Ca released from 24 to 48 hours was considered for comparisons of resorption. Resorption was expressed as % ^{45}Ca released/μg DNA/ 24-48 hours.

The effect of PTH on resorption was examined at a phosphate concentration of 1 mM.

Collagen production culture

Incubation media for cultures to be assayed for collagen production included 4μCi/ml 2,3-^{3}H-proline (New England Nuclear) and 0.1 mg/ml ascorbic acid. Cells were seeded in sterile microtiter wells and immediately covered with 1.0 ml incubation medium or seeded on the surfaces of devitalized calvaria as described above.

At the termination of culture, to precipitate soluble collagen from the media, 0.5 ml 15% trichloroacetic acid was added to each culture well. The well was then scraped with a Teflon policeman and the contents transferred to centrifuge tubes. Contents were centrifuged at 5000 rpm for 5 minutes, rinsed with 15% trichloroacetic acid, and recentrifuged. The supernatants were replaced with 6.0 N HCl. The samples were hydrolyzed at 110° C for 24 hours, dried under N_2, and resuspended in 1.0 ml deionized, distilled water.

Radiolabeled hydroxyproline was separated using thin layer chromatography. Samples of hydrolyzated well contents were spotted on activated Silica Gel G thin layer plates. Standards of non-

radioactive proline and hydroxyproline were spotted over the hydrolyzate sample so as to facilitate identification of proline and hydroxyproline bands on the developed plate. Silica Gel G plates were poured to a thickness of 4 mm in the laboratory. The plates were activated by heating at 110° C for 30 minutes. The thin layer plates were developed in a 3:1 n-propanol:water solvent system (47). Following development the plates were sprayed with a 1% ninhydrin solution and heated at 110° C until amino acid bands became maximally visible. Only hydroxyproline counts were used as an assessment of collagen formation.

Collagen formation is expressed as cpm hydroxyproline/μg DNA/ 24 hours of culture.

The collagen assay was tested using cultured bone cells. Equal numbers of isolated bone cells were seeded at 1×10^6 cells/ well in 12 microtiter wells and incubated in varying mediums for 24 hours. Four wells contained 100μg/ml alpha, alpha'-dipyridyl, an iron binding agent; four wells contained 100μg/ml cycloheximide, and four wells were used as controls.

The effect of PTH on collagen synthesis was examined at a phosphate concentration of 1 mM. The effect of inorganic ortho-phosphate concentration on collagen synthesis in peak III cells was examined at phosphate concentrations ranging from 0.5-4.0 mM.

PTH induced biochemical changes

To assess the effect of PTH on peak III cells, two experiments were devised. In each experiment, peak III cells were divided: one

half were cultured in the presence of PTH; the other half were cultured as controls.

Both PTH-treated and control cell populations were re-run through the free flow apparatus after 48 hours of culture. The cells were cultured in the presence of ^{3}H-thymidine (New England Nuclear). They were removed from cell culture following incubation in the collagenase containing digestion media initially used to isolate the cells from fetal rat calvaria. The termination of the cell cultures was staggered so as to allow equal times for cell recovery between digestion and re-run. Following the FFE runs, the cells were lysed, their DNA content assayed to determine the pattern of deflection.

In the second experiment, cells from the PTH treated and control groups were assayed for acid phosphatase. Acid phosphatase was measured by the method of Bessey et al.(48) utilizing p-nitrophenyl phosphate as substrate.

Parathyroid Hormone and DNA determinations

The parathyroid hormone (1800 units/mg) employed in this work was the gift of Professor Howard Rasmussen. It was dissolved in 10 mM HCl, 145 mM NaCl with 1.0 mg/ml bovine serum albumin and 50 mM β-mercaptoethanol.

The content of DNA in cell cultures was determined by the method of Puzas and Goodman(46).

Data analysis

Data are represented as mean ± standard error of the mean. Statistical comparisons are made by Student's t test. Levels of significance are noted in the appropriate tables.

Results

Collagen production

Collagen was assayed by quantitating ^{3}H-hydroxyproline converted from ^{3}H-proline. A solvent system which would carry hydroxyproline further up the Silica Gel G plates than proline was chosen to avoid any contamination secondary to lag. Using 3:1 n-propanol: water, R_f values for hydroxyproline and proline were consistantly observed to be 0.50 and 0.38, respectively.

Bone cell cultures incubated in the presence of alpha, alpha'-dipyridyl and cycloheximide produced insignificant amounts of collagen (Table 1). A repeated determination of a single sample showed a standard deviation less than one sixth that of samples taken from control wells.

Isolated, separated bone cells from peak III produce significantly more collagen than cells from peak I (Table 2). The difference in collagen production between peak II and peak III is insignificant. Peak I cells, osteoclasts and/or preosteoclasts, would be expected to produce less collagen than peak III cells, osteoblasts and preosteoblasts. That the fibroblasts of peak II produce as much collagen as peak III cells is not surprising.

The effect of parathyroid hormone on the collagen synthesis of the different cell types was investigated (Table 2). PTH was added to the incubation medium of half the wells; half were maintained as controls. Collagen synthesis was significantly reduced by PTH only in peak III cells. There were no other significant changes.

The effect of varying concentrations of inorganic orthophosphate on collagen production of peak III cells was measured (Figure 5). Cells were incubated in phosphate concentrations varying from 0.5 mM to 4.0 mM. Collagen production was greatest in cells grown at 2.0 and 4.0 mM, which were not significantly different. Cells growing at 2.0 mM produced significantly more collagen than those growing at 1.0mM. Therefore, collagen production was maximized for peak II cells at 2.0 mM phosphate.

Resorption

Resorption induced by separated bone cells was greatest when peak I cells and smallest when peak III cells were incubated with devitalized bone (Table 3). The difference between peak I cells, on one hand, and peaks II and III cells, on the other, is significant. The resorptive difference between peak II and peak III cells is insignificant. As would be expected, the osteoclasts and pre-osteoclasts resorb more bone than the osteoblasts and preosteoblasts.

The hormonal responsiveness of the separated bone cells to PTH was examined using the resorption assay (Table 3). Neither peak I nor peak II cells were significantly stimulated from control values. Peak III cells were significantly stimulated to increase resorption. The resorption of the PTH stimulated peak III cells is less than, but not significantly different from, the peak I control resorption cultures.

PTH-induced biochemical changes

To determine if PTH had an effect on the differentiation of peak III cells while in culture, experiments were designed to identify changes in cell activity of the marker enzyme acid phosphatase and changes in the cell surface charge density.

Peak III cells treated with PTH had a significantly different acid phosphatase activity from control treated cells, an increase of 45%. Although elevated from controls, this increased activity of acid phosphatase did not approximate the acid phosphatase activity of cells as determined by Puzas (Table 4). It is remarkable that PTH had no effect on cell proliferation as determined by DNA content.

To identify a change in the cell surface charge density of peak III cells cultured in the presence of PTH, cells were re-run on the FFE apparatus. Fractions were assayed (Figure 6) for cell content by determining counts of ^{3}H-thymidine. The peak III cells, after 48 hours incubation with PTH, showed significant migration toward peak I; in fact, there was some overlap with fractions (30-31) previously identified with peak I cells (Figure 4). The control treated peak III cells showed no migration after 48 hours of cell culture relative to previously identified fractions (33-39) of peak III.

Discussion

Isolated bone cells have provided an interesting and informative system in which to study the biology of bone. Cells isolated by enzymatic digest have been shown to retain differentiated functions, including collagen synthesis and resorptive capacity. However, because the enzymatic digest produces a mixture of bone cell types, studies have been hampered by an inability to discern which cells respond to various stimuli.

Bone cells in different stages of differentiation and/or having different functions would be expected to have different proteins on their cell surfaces. Cell surface charge density differences have been exploited as a means of separating cell types. Separating bone cells according to surface charge density has resulted in the isolation of three populations of bone cells. Puzas et al. characterized the cells using marker enzymes, hormonal sensitivity, and morphometric correlation of peak populations with populations of bone cells in intact calvaria. Data characterizing the cells according to function, i.e. collagen production and matrix resorption, are presented here. Morphometric studies of the cells separated by FFE have not yet been undertaken.

Peak I cells had the greatest migration in FFE and were, therefore, the cells with the most negative cell surface charge density. Their proportion relative to the total number of cells recovered from FFE correlated with the proportion of osteoclast and osteoclast-like cells seen in morphometric studies of intact fetal calvaria(34). These cells respond to parathyroid hormone and calcitonin with increases in cellular cyclic AMP(45). Osteoclast

marker enzymes, acid phosphatase and carbonic anhydrase, were found at higher levels in peak I cells. Furthermore, when cultured on pre-labeled, devitalized bone, peak I cells release more mineral than cells from the other peaks. And, consistant with the function of osteoclasts, peak I cells produce less collagen. These data are consistant with peak I cells being osteoclasts and osteoclast-like cells by biochemical and functional characteristics.

Peak II cells were shown to be the first cells released by enzymatic digestion of fetal rat calvaria. After 20 minutes of incubation with collagenase, cells released from calvaria were shown to migrate during FFE to the position of peak II cells. Morphologic study of the partially digested calvaria showed an absence of fibroblasts and loose connective tissue cells. The peak II cells were not responsive to PTH or CT(45). They resorbed the least amount of mineral from devitalized, pre-labeled calvaria. Consistant with one of the capabilities of fibroblasts, the peak II cells produced significant amounts of collagen.

Peak III cells migrated least during FFE, having the least negative cell surface charge density. Their proportion relative to the total number of cells collected from FFE correlated with the proportion of osteoblasts and pre-osteoblasts seen in morphometric studies of intact fetal rat calvaria(34). The peak III cells are not responsive to CT, as determined by cyclic AMP elevations, but were most responsive to PTH. Unlike peak I cells, peak III cells contained the least amount of osteoclast marker enzymes, acid phosphatase and carbonic anhydrase. Functionally, they resorbed devitalized, pre-labeled bone less well than peak I cells and

produced the greatest amounts of collagen.

The effect of inorganic orthophosphate concentration on collagen synthesis in peak III cells was in agreement with studies done in tissue culture (supra vide). Increasing the concentration of phosphate beyond 2.0 mM did not increase the amount of collagen produced. There was a significant difference in collagen production of cells incubated at 1.0 mM phosphate and those at 2.0 mM. This finding suggests that the effect of phosphate on collagen production is a stimulatory effect on osteoblasts and pre-osteoblasts. Raisz and Niemann(23) showed that increasing phosphate concentration in tissue culture media decreased the mineral loss. Presumably, such an effect would be mediated by a decrease in osteoclast activity. However, resorption of bone was not assayed in peak III cell cultures at concentrations greater than 1.0 phosphate. Therefore, a conclusion that the effect of phosphate on collagen production of peak III cells is a stimulatory effect on collagen producing cells is unjustified. Perhaps increasing the concentration of phosphate in resorption cultures of peak III cells would have decreased mineral release.

The effect of PTH on collagen production of peak III cells was also in agreement with studies done in tissue culture (supra vide). Collagen production was significantly inhibited only in peak III cells. However, interpretation of this result is unclear: collagen synthesis may have been inhibited by PTH, or synthesized collagen may have been destroyed.

When cells cultured on devitalized, pre-labeled fetal rat calvaria were incubated in the presence of PTH, only peak III cells showed a significant increase in resorption. The increased resorption

of peak I cells was not significant. This result was surprising because the most obvious changes which occur in bone in vivo following PTH treatment are an increase in the activity and number of osteoclasts.

A review of the morphometric data used by Puzas et al. to correlate the proportion of cells in peak III to the proportion of cells making up osteoblasts and pre-osteoblasts in intact calvaria permits a different interpretation. Puzas et al. assumed that osteoprogenitor cells would mature to become osteoblasts. Hence peak III contains osteoblasts and osteoprogenitor cells. This semantic distinction is important since osteoprogenitor cells could become either osteoblasts (as was assumed by Puzas) or osteoclasts. The stimulatory effect of PTH on the resorptive capacity of peak III cells might be explained by an induction of functional differentiation of osteoprogenitor cells and/or their cellular progeny.

Acid phosphatase was found to have greater activity in peak III cells incubated with PTH than in peak III cells incubated as controls. The activity of acid phosphatase/μg of DNA was not as great in the PTH stimulated peak III cells as in control cultures of peak I cells as determined by Puzas. This descrepancy might be explained in several ways. Because peak III is composed of at least two cell types, perhaps only one cell type responds to PTH with increased acid phosphatase activity. When acid phosphatase activity is expressed on the basis of DNA content, the denominator may represent cells which are not increasing their content of acid phosphatase-- hence, lowering the recorded activity per cell. Or, independent (but not exclusive) of the assumption that cell types respond differently to PTH, the

period of incubation may not be long enough for complete differentiation of osteoprogenitor cells and/or their cellular progeny.

When peak III cells were incubated with PTH and re-run through free flow electrophoresis, they migrated in a different pattern than peak III cells incubated as controls. Peak III cells from control cultures migrated with the same mobility as cells not previously exposed to FFE. However, some peak III cells from PTH treated cultures developed a more negative surface charge density and, consequently, migrated closer to peak I cells. It should be noted that not all of the cells exhibit increased migration toward the positive electrode. It is reasonable to suggest that treatment with PTH induced a change in one cellular component of peak III, and not in the others. Perhaps a longer incubation period and/or manipulation with other hormonal or ionic stimuli might result in a separation of the cellular components of peak III.

In summary, three experiments support the idea that PTH induces in ulture at least one cell type of peak III to take on characteristics associated with osteoclasts: (1) increased resorption; (2) increased activity of acid phosphatase; and (3) more negative cell surface charge density. The decreased amount of collagen found in peak III cultures incubated with PTH (supra vide) can not, therefore, be taken as evidence that PTH inhibits the collagen production of peak III cells. PTH may inhibit collagen production, but the increased resorptive capability of peak III cells incubated with PTH precludes such an interpretation from this experiment.

The PTH-induced differentiation of a population of peak III cells supports the theory that some osteoclast may have a precursor

in skeletal tissue. Young(49) found tritiated thymidine injected into six day old rats localized in osteoprogenitor cells after 3-4 hours, osteoblasts after 9-10 hours, and metaphyseal osteoclasts after 9-10 hours. The conclusion that osteoprogenitor cells are the precursors of osteoclasts has been attacked. Hall(50) argues that because all dividing cells in the rats would have been labeled by the tritiated thymidine, the osteoclasts may have arisen from blood-borne precursors.

Data reported here support the theory that osteoclast may arise from progenitor cells in bone. Furthermore, evidence from isolated cells in culture suggests peak III cells can be induced to resorb more bone than monocytes. The PTH-treated peak III cells resorbed as much bone as peak I control cells. When monocytes were cultured on pre-labeled, devitalized bone(19), resorption was one third as great as that shown by a mixed population of isolated calvarial cells. PTH did not stimulate resorption in the cultured monocytes, although the cells were in culture for up to 8 days. Perhaps other signals, not present in this resorption assay, are necessary for monocytic transformation.

Although these data are consistant with the presence of a progenitor cell for osteoclasts in bone, they do not elucidate the origin of the progenitor cell. Whether the progenitor cell arises in bone or represents a blood-borne precursor is unknown. Furthermore, the presence of an osteoclast precursor in bone does not exclude the participation of blood-borne cells in the <u>in vivo</u> evolution of an osteoclast.

Additional evidence for the presence of an osteoclast precursor in bone has recently been reported by Raisz and co-workers(51). Fetal rat calvaria were incubated in organ culture in the presence of PTH and as controls. Histologic examination of the calvaria showed an increased number of osteoclasts in the PTH-treated calvaria. In the absence of a blood supply, osteoclasts must have originated from precursors in bone.

Groups of bone cells separated by the method of sequential enzymatic digestion(37,38,39,40) are different from the subpopulations separated by FFE. Although similar in their cyclic AMP responses to parathyroid hormone and calcitonin, "CT" (osteoclasts) and "PT" (osteoblasts) cell groups differ from peak I and peak III cells. In cell culture "PT" cells did not increase their content of acid phosphatase in response to PTH(39) and "CT" cells, not "PT" cells, showed the greatest increase in bone resorption after incubation with PTH(40). It is possible that osteoprogenitor cells are separated with osteoclasts in this method, rather than with osteoblasts as they would seem to be with free flow electrophoresis.

The use of FFE to follow the PTH-induced <u>in vitro</u> differentiation of at least one cellular component of peak III represents a new, and potentially powerful tool in the study of bone cell differentiation. That cells in different stages of development can be separated on the basis of cell charge has been demonstrated: the electrophoretic distribution of hemopoietic stem cells separated the cells into two stages of development(52). This is the first known report of <u>in vitro</u> differentiation assessed using the FFE separation technique.

Figure 1: Schematic representation of free flow electrophoresis

An electrolyte solution flows across the lines of an electric field. Isolated bone cells were injected continuously from the starting port. The cells were deflected in the FFE chamber according to surface charge density. Cells of different surface charge densities arrived at different ports at the bottom of the chamber. The angle α represents the angle of deflection.

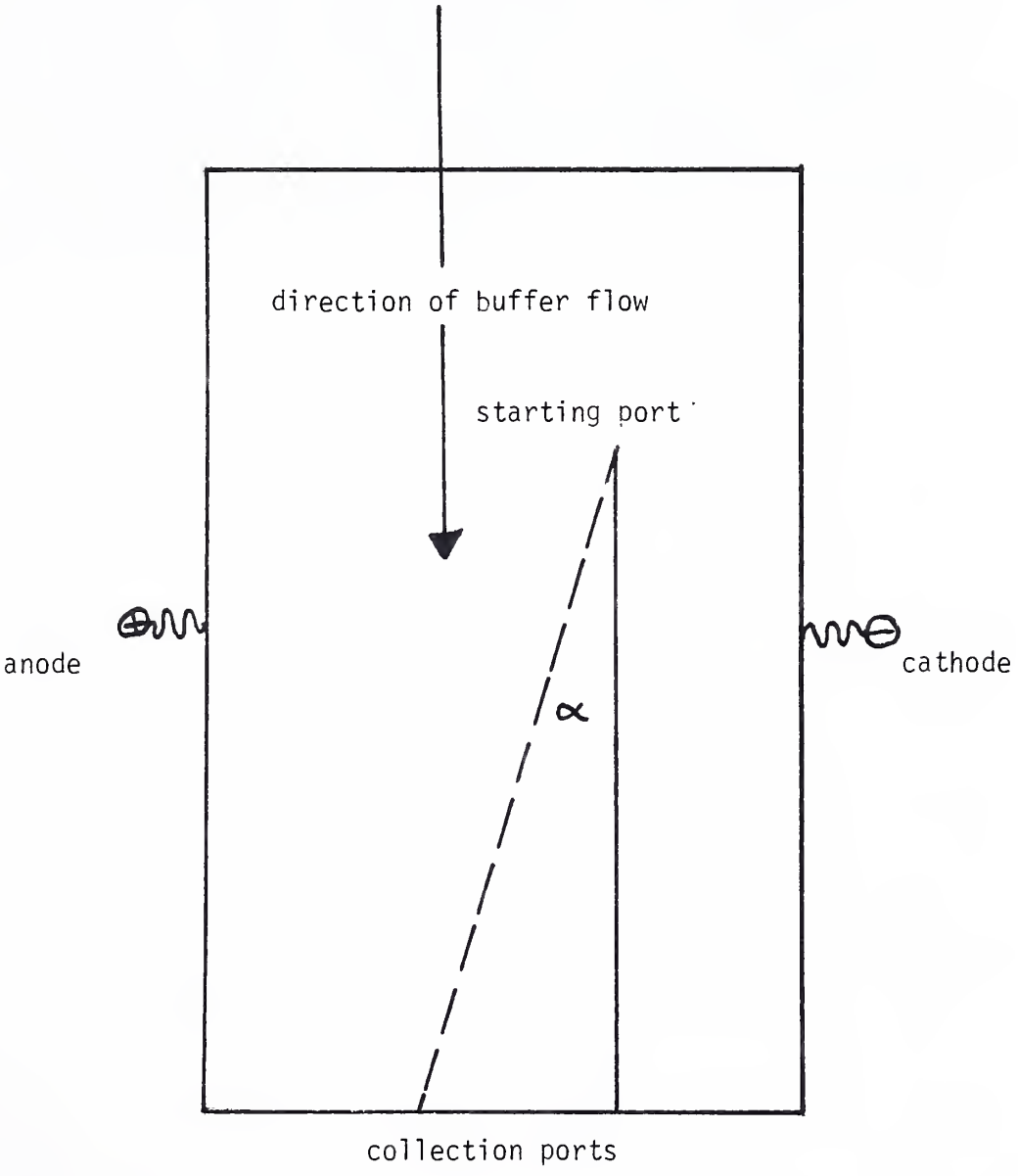

direction of buffer flow
starting port
anode
cathode
α
collection ports

Figure 1

Figure 2: Isolated bone cells separated using free flow electrophoresis.

Patterns obtained when bone cells isolated from fetal rat calvaria are subjected to free flow electrophresis. The (+) represents the anode and the (-) the cathode of the separation chamber. Cells were introduced into the chamber over fraction #72. Panel A is the pattern obtained when cells recovered from a 0-120 minute collagenase digestion of the calvaria are subjected to FFE. Panel B is obtained when cells recovered from a 0-20 minute digestion are subjected to FFE. Panel C is obtained when cells recovered from 20-120 minute digestion were subjected to FFE.
(Taken from Puzas et al.(34))

Figure 2

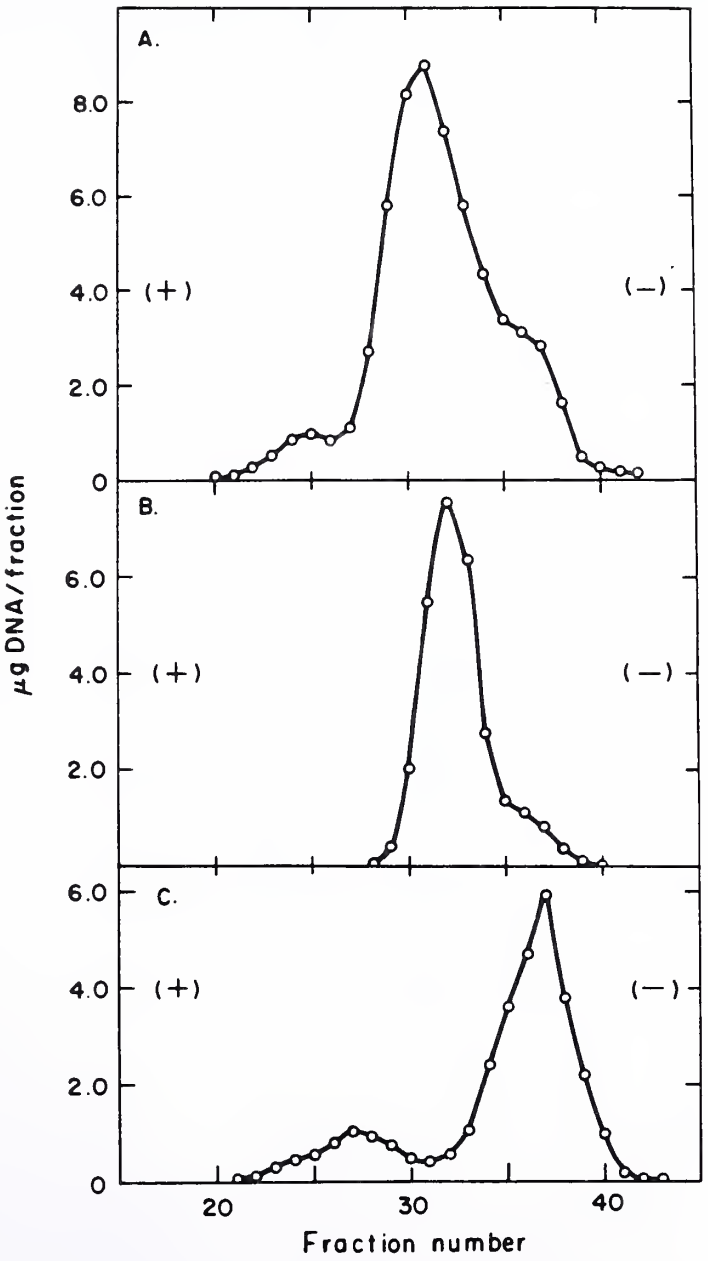


Figure 3: Alkaline and acid phosphatase in isolated bone cells separated using FFE.

The content of acid and alkaline phosphatase from the cells in each fraction of FFE run. The (o) indicates the electrophoretic pattern of cells obtained from a 0-120 minute digestion of the calvaria; (▲) the amount of phosphate released from p-nitrophenyl phosphate per μg DNA at pH 10.5; and (●) the amount of phosphate released from p-nitrophenyl phosphate per μg DNA at pH 4.8. (Taken from Puzas et al.(34))

Figure 3

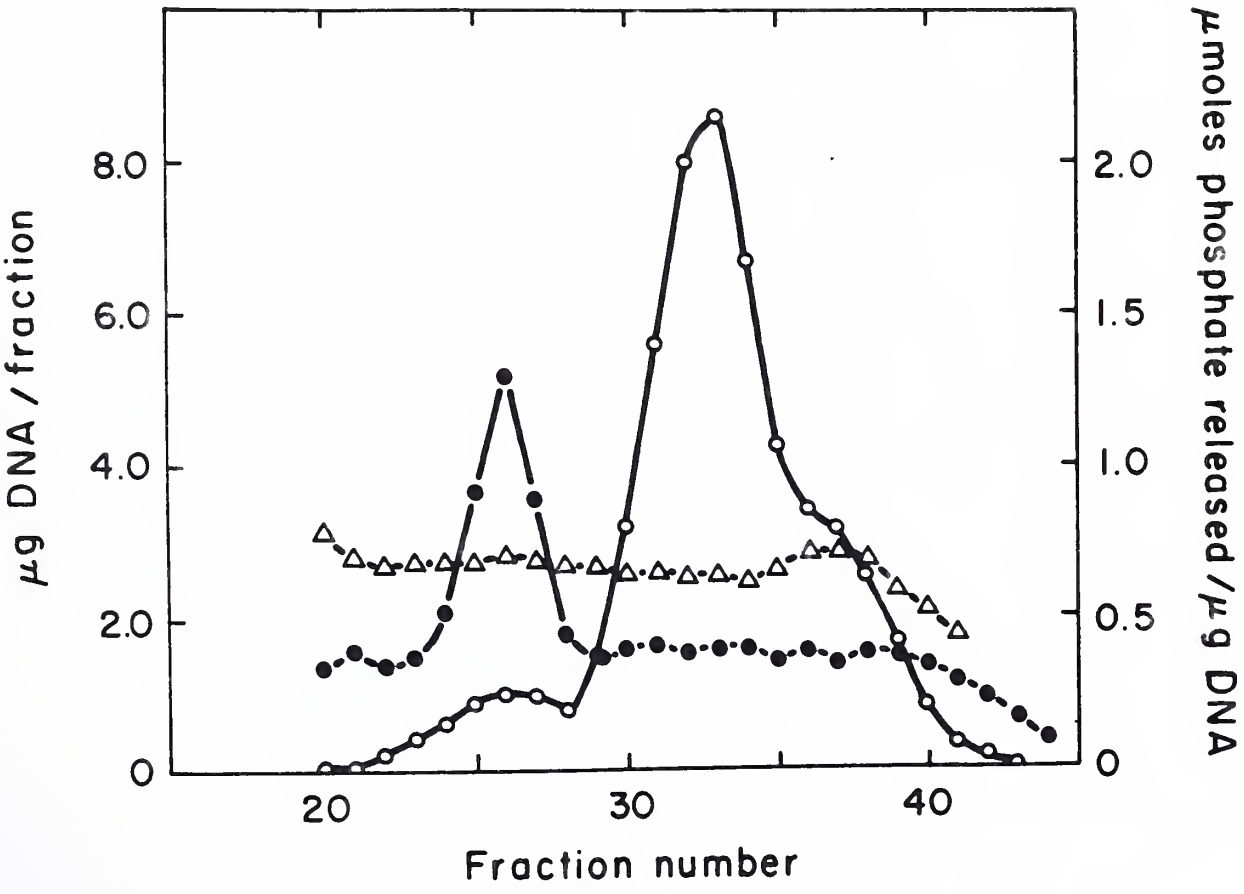

μg DNA / fraction
μmoles phosphate released / μg DNA
Fraction number
0
2.0
4.0
6.0
8.0
0
0.5
1.0
1.5
2.0
20
30
40

Figure 4: Specific binding of ^{3}H-acetazolamide to cells prior to separtion using FFE.

The specific binding of ^{3}H-acetazolamide to cells recovered from a 20-120 minute collagenase digestion of fetal rat calvaria. The freshly isolated cells were incubated with 4 μCi/ml of ^{3}H-acetazolamide for 20 minutes and then washed in isotope-free media for 40 minutes before being subjected to FFE. The (o) represents the cell profile expressed as μg of DNA and (●) represents the binding of ^{3}H-acetazolamide as cpm/μg DNA.

(Taken from Puzas et al.(34))

Figure 4

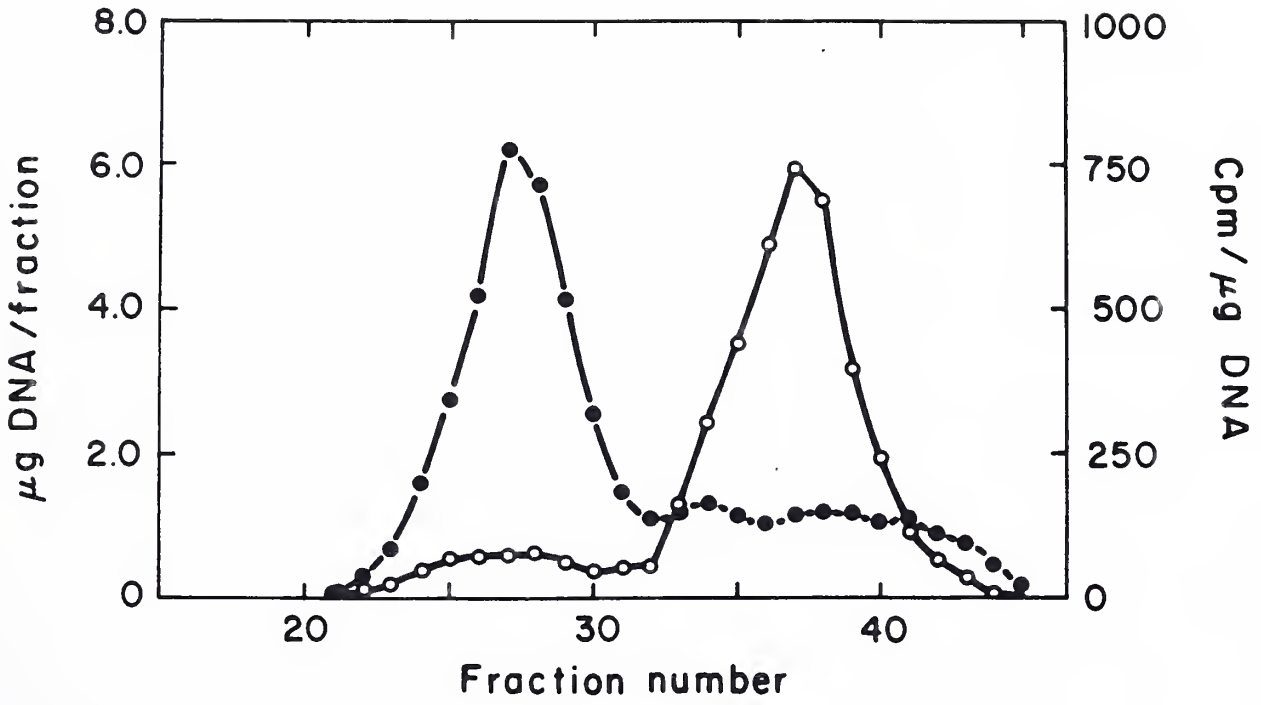
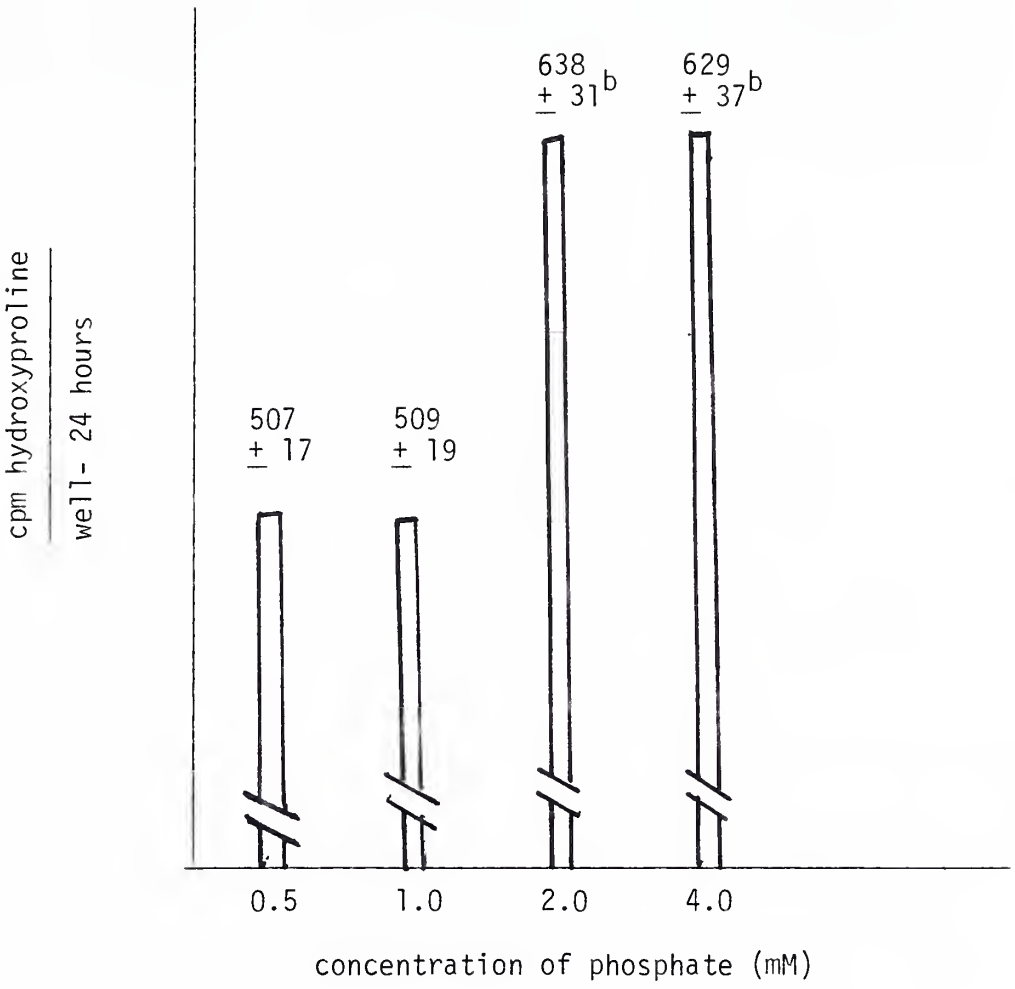

8.0
6.0
4.0
2.0
0
μg DNA/fraction
1000
750
500
250
0
Cpm/μg DNA
20
30
40
Fraction number

Figure 5: Effect of orthophosphate concentration on collagen production of peak III cells.

Bone cells isolated from fetal rat calvaria were subjected to FFE. Equal numbers of peak III cells were seeded in microtiter wells and incubated at 0.5, 1.0, 2.0, and 4.0 mM phosphate.

Figure 5[a]

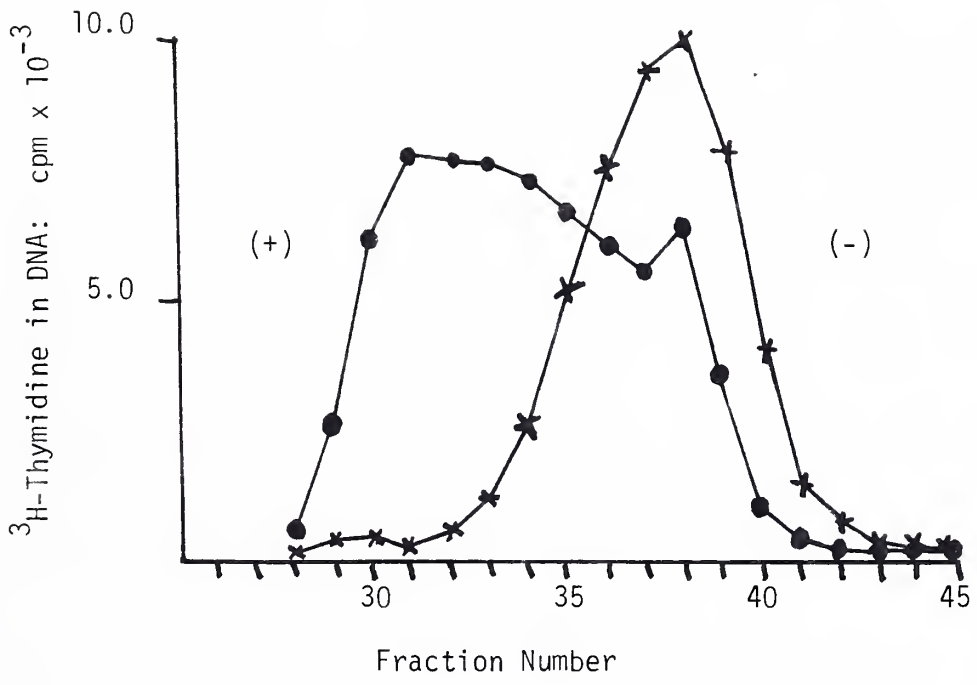


a) Values represent mean ± SEM; n=4.
b) Significantly different from phosphate = 1.0 mM with $p < .02$.

Figure 6: Effect of PTH on the cell surface charge density of peak III cells as determined by FFE re-run.

Bone cells were isolated from fetal rat calvaria and subjected to FFE. Peak III cells were seeded in microtiter wells. Cell cultures were incubated in the presence of 100 ng/ml PTH and as controls. Cell cultures were re-suspended following incubation with the collagenase containing digestion medium. Cell populations were re-run on FFE.

Figure 6



● PTH-treated cells

× Control cells

Table 1: Effect of alpha, alpha'-dipyridyl and cycloheximide on collagen production of a mixed bone cell population

Fetal rat calvaria were digested with collagenase for 120 minutes. Equal numbers of the isolated cells were seeded in microtiter wells. Cells were incubated in the presence of 100 μg/ml alpha, alpha'-dipyridyl, 100 μg/ml cycloheximide, or as controls. Concentration of inorganic orthophosphate was 1.0 mM.

Table 1: Effect of alpha, alpha'-dipyridyl and cycloheximide on collagen production of a mixed bone cell population[a]

	cpm hydroxyproline / well- 24 hours	significance from control (p value)
control	1093 ± 108	
alpha, alpha'-dipyridyl	74.7 ± 8.8	$p < .001$
cycloheximide	89.6 ± 10.7	$p < .001$

a) Values represent mean ± SEM; n=4.

Table 2: Effect of PTH on collagen production of separated bone cell types

Isolated bone cells were separated using FFE and seeded in microtiter wells. Cell cultures were incubated in the presence of 100 ng/ml PTH and as controls.

Table 2

	cpm hydroxyproline / μg DNA- 24 hours[a]	
	control	PTH
Peak I (fractions 22-24)	478 ± 50	449 ± 23[b]
Peak II (fractions 28-36)	858 ± 86[d]	905 ± 72[b]
Peak III (fractions 37-39)	887 ± 23[d]	748 ± 26[c]

a) Values represent mean ± SEM; n=4.
b) Not significantly different from control.
c) Significantly different from control with $p < .01$.
d) Significantly different from control peak I value with $p < .001$.

Table 3: Effect of PTH on mineral release by separated bone cell types.

Bone cells isolated from fetal rat calvaria were subjected to FFE and seeded on pre-labeled, devitalized bone. Cell cultures were incubated in the presence of 100 ng/ml PTH and as controls.

Table 3

	% ^{45}Ca released / µg DNA- 24-48 hours[a]		
	control	PTH	PTH / control
Peak I (fractions 22-24)	4.4 ± .4[d]	5.4 ± .5[b]	1.23
Peak II (fractions 28-30)	2.88 ± .2	3.2 ± .4[b]	1.11
Peak III (fractions 37-39)	2.5 ± .3	3.9 ± .6[c]	1.56

a) Values represent mean ± SEM; n=4.
b) Not significantly different from control.
c) Significantly different from control with $p < .05$.
d) Not significantly different from PTH stimulated peak III.

Table 4: Effect of PTH on acid phosphastase activity and DNA content in peak III cells.

Bone cells were isolated from fetal rat calvaria and subjected to FFE. Peak III cells were seeded in microtiter wells. Cell cultures were incubated in the presence of 100 ng/ml PTH and as controls. Acid phosphatase activity was assayed following the method of Bessey et al.(48).

Table 4

	μm PO_4 liberated / μm DNA- 30 minutes	μg DNA / well
Peak III (control)	.203 ± .005	1.65 ± .17
Peak III (PTH)	.294 ± .009[b]	1.56 ± .08[c]
Peak I (control)[e]	.70 ± .09[d]	*

a) Values represent mean ± SEM; n=7-8.
b) Significantly different from control with $p < .001$.
c) Not significantly different from control.
d) Significantly different from Peak III (PTH) with $p < .001$.
e) Data from Puzas et al.(45).
*) Data not available.

Acknowledgments

The author would like to acknowledge the teaching talent and superior athletic skills of Dr. Ed Puzas. Only with the personal coaching of Dr. Puzas was I able to go the distance with the rats.

David "B.P." Goodman '64 made the laboratory a place of serious work and serious play. His professional contribution, particularly on the collagen assay, is gratefully acknowledged.

Roland Baron and Agnes Vignery helped sustain my interest in bone despite a long, first summer of sectioning methyl metacrylate.

And, of course, none of this work would have been possible without the support and assistance of my advisor and friend, Professor Howard Rasmussen. (His critical readings of this thesis, and suggestion that the first two drafts be used as Guiac testing paper, were truely invaluble.) Dr. Rasmussen has been more than a faculty advisor-- he has been an authentic and honest friend. He's a great thinker and a great guy.

Finally, I must acknowledge the support of Jean and her entourage of fictional friends. Together, they made medical school tolerable.

Abstract

Bone cells, isolated from fetal rat calvaria by collagenase digestion, have been separated into three populations using free flow electrophoresis. Puzas et al. characterized the cells using marker enzymes, hormonal sensitivity, and morphometric correlation with intact fetal rat calvaria. Peak I cells were identified as osteoclasts and pre-osteoclasts, peak II cells as fibroblasts, and peak III cells as osteoblasts and pre-osteoblasts. Functional characterization of the cells by assessment of collagen production and resorption of devitalized bone matrix is presented here.

Peak I cells were found to release significantly more mineral from pre-labeled, devitalized fetal rat calvaria than peak II or peak III cells. Peak II and peak III cells produced significantly more collagen than peak I cells. When cell cultures were incubated in the presence of PTH, significantly less collagen was produced by peak III cells. However, PTH significantly increased resorption of devitalized bone only in peak III cells. Suspicion that PTH was inducing a progenitor cell population in peak III to take on the characteristics of an osteoclast was tested with two experiments. When peak III cells were incubated with PTH and as controls, (1) the PTH-treated cells increased the activity of cellular acid phosphatase; and (2) the PTH-treated cells shifted their migratory pattern upon re-run through free flow electrophoresis. These data are interpreted as supportive of the presence of an osteoclast progenitor cell in bone tissue.

Bibliography

1) Peck, W.A., S.J. Birge, and S.A. Fedak, Bone Cells: Biochemical and biological studies after enzymatic isolation. Science 146: 1476-1477, 1964.

2) Dziak, R., and J.S. Brand, Calcium transport in isolated bone cells: Bone isolation procedures. J Cell Physiol 84: 75, 1974.

3) Frost, H.M., Dynamics of bone remodeling. In: Bone Biodynamics. H.M. Frost, ed. Little, Brown & Co., Boston, 1964.

4) Bassett, C.A.L., Biologic significance of piezoelectricity. Calcified Tissue Res 1: 252-272, 1968.

5) Horton, J.E., L.G. Raisz, H.A. Simmons, J.J. Oppenheim, S.E. Metgenhagen. Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes. Science 177: 793-795, 1972.

6) Mundy, G.R., Bone resorbing activity in supernatants from lymphoid cell lines. N Engl J Med 290: 867-871, 1974.

7) Dietrich, J.W., J.M. Goodson, and L.G. Raisz, Stimulation of bone resorption by various prostaglandins in organ culture. Prostaglandins 10:231-238, 1975.

8) Raisz, L.G., C.L. Trummel, and H. Simmons, Induction of bone resorption in tissue culture. Endocrinology 90:744-752, 1972.

9) Raisz, L.G., C.L. Trummel, M.F. Holick, and H.F. DeLuca, 1,25-dihydroxycholecalciferol: A potent stimulator of bone resorption in tissue culture. Science 175: 768-769, 1972.

10) Rasmussen, H., and P. Bordier, The Physciological and Cellular Basis of Metabolic Bone Disease, Williams and Wilkins Co., Baltimore, p. 146-148, 1974.

11) Peck, W.A., J. Carpenter, K. Messinger, and D. Debra, Cyclic AMP in isolated bone cells: Response to low concentrations of PTH. Endocrinology 92: 692-697, 1973.

12) Gothlin, G., and J.L.E. Ericcson, On the histogenesis of the cells in fracture callus. Referenced in (50).

13) Buring, K., On the origin of cells in heterotopic bone formation. Clin Orthop and Rel Res 110: 293-302, 1975.

14) Vaes, G., On the mechanism of bone resorption. J Cell Biol 39:676-697, 1968.

15) Rasmussen and Bordier, p. 98.

16) Puzas, J.E. and J.S. Brand, Collagenolytic activity from isolated bone cells. Biochim Biophy Acta 429: 964-974, 1976.

17) Puzas, J.E., and J.S. Brand, Parathyroid hormone stimulation of collagenase secretion by isolated bone cells. Endocrinology 104: 669, 1979.

18) Raisz, L.G., Bone resorption in tissue culture: Factors influencing the response to PTH. J Clin Invest 44: 103-116, 1965.

19) Mundy, G.R., A.J. Altaman, M.D. Gondek, and J.G. Bandelin, Direct resorption of bone by human monocytes. Science 196:1109-1111, 1977.

20) Tatevossian, A., Effect of parathyroid extract on blood calcium and osteoclast count in mice. Calcif Tissue Res 11: 251-259, 1973.

21) Holtrop, M.E., L.G.Raisz, and H.A. Simmons, The effects of parathyroid hormone, colchicine, and calcitonin on the ultrastructure and activity of osteoclasts in organ culture. J Cell Biol 60: 346-355, 1974.

22) Brand, J.S., and L.G. Raisz, Effects of thyrocalcitonin and phosphate ion on the PTH stimulated resorption of bone. Endocrinology 90: 479-487, 1972.

23) Raisz, L.G., and I. Niemann, Effect of phosphate, calcium, and magnesium on bone resorption and hormonal responses in tissue culture. Endocrinology 85: 446-452, 1969.

24) Grant, M.E., and D.J. Prockop, The Biosynthesis of collagen (second of three parts). N Engl J Med 286: 242-249, 1972.

25) Berg, R.A., and D.J. Prockop, Affinity column purification of protocollagen proline hydroxylase. J Biol Chem 248: 1175, 1973.

26) Peterkofsky, B. and R. Diblasio, Anal Biochem 66: 279, 1975.

27) Dietrich, J.W., E.M. Canalis, D.M. Maina, and L.G. Raisz, Hormonal control of bone collagen synthesis in vitro: Effects of PTH and calcitonin. Endocrinology 98: 943-949, 1976.

28) Peterkofsky, B., and R. Diegelmann, Use of a mixture of proteinase free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 10: 988-994, 1971.

29) Birge, S.J., and W.A. Peck, Collagen Synthesis by isolated bone cells. Biochem Biophys Res Commun 22: 532, 1966.

30) Peck, W.A., S.J. Birge, and J. Brandt, Collagen synthesis by isolated bone cells: Stimulation by ascorbic acid in vitro. Biochim Biophy Acta 142: 512-525, 1967.

31) Asher, M.A., C.B. Sledge, and M.J. Glimscher, The effect of inorganic phosphate on the rates of collagen formation and degradation in bone and cartilage in tissue culture. J Clin Endocrinol Metab 38: 376-390, 1974.

32) Miller, S.S., A.M. Wolf, and C.D. Arnaud, Bone cells in culture: Morphologic transformation by hormones. Science 192: 1340-1343, 1976.

33) Altman, A.J., J.G. Bandelin, J.H. Dominguez, G.R. Mundy, Differentiation of isolated calvarial cell into a mature hetero genous bone cell population in culture. Metabolic Bone Disease and Rel Research 1: 75-79, 1978.

34) Puzas, J.E., A. Vignery, and H. Rasmussen, Isolation of specific bone cell types by free flow electrophoresis. Calcif. Tiss Intl 27: 263, 1979.

35) Smithe, D.M., C.C. Hohnston, and A.R. Severson, Studies of the metabolism of separated bone cells. Calcif Tiss Res 11: 56-69, 1973.

36) Yagiela, J.A., and D.M. Woodbury, Enzymatic isolation of osteoblasts from fetal rat calvaria. Anat Record 188: 287-305, 1977.

37) Wong, G., and D.V. Cohn, Separation of parathyroid hormone and calcitonin sensitive cells from nonresponsive bone cells. Nature 252: 713-715, 1974.

38) Wong, G., and D.V. Cohn, Target cells in bone for PTH and calcitonin are different: Enrichment for each cell type by sequential digestion of mouse calvaria. Proc Nat Acad Sci 72: 3165-3171, 1975.

39) Luben, R., G. Wong, and D.V. Cohn, Biochemical characterization with PTH and calcitonin of isolated bone cells: Provisional identification of osteoclasts and osteoblasts. Endocrinolgy 99: 526-534, 1976.

40) Luben, R., G. Wong, and D.V. Cohn, PTH stimulated resorption of devitalized bone by cultured osteoclast type bone cells. Nature 265: 629-630, 1976.

41) Ambrose, E.J., Cell Electrophoresis, Churchill, London, 1966.

42) Hannig, K., Separation of cells and particles by continuous free flow electrophoresis. In: Techniques of Biochemical and Biophysical Morphology, Vol. I, Eds. D. Glick and R.M. Rosebaum, Wiley and Sons, New York, 1972.

43) Wergedal, J.E. and D. Baylink, Distribution of acid and alkaline phosphatase activity in undermineralized sections of the rat tibial diaphysis. J Histochem and Cytochem 17:799-806, 1969.

44) Gay, C. and W. Mueller, Carbonic anhydrase and osteoclasts: Localization by labeled inhibitor autoradiography. Science 183: 432-434, 1974.

45) Puzas, J.E., and H. Rasmussen, unpublished results.

46) Puzas, J.E., and D.B.P. Goodman, A rapid assay for cellular deoxyribonucleic acid. Anal Biochem 86: 50-55, 1975.

47) Randerath, K., Thin Layer Chromatography, ed. 2, Academic Press, New York, 1966, p.110.

48) Bessey, O., and O. Lowry, and M. Brock, A method for the rapid determination of alkaline phosphatase. J Biol Chem 164:321,1946.

49) Young, R.W., Cell proliferation and specialization during endochondral osteogenesis in young rats. J Cell Biol 14:357-370, 1962.

50) Hall, B.K., The origin and fate of osteoclasts, Anat Rec 183: 1-12, 1975.

51) Holtrop, M.E., and L.G. Raisz, Comparison of the Effects of 1,25-Dihydroxycholecalciferol, PG E2, and OAF with PTH on the ultrastucture of osteoclasts in cultured long bones of fetal rats. Calcif. Tissue Intl 29: 201-205, 1979.

52) Zeiller, K., J.C.F. Schubert, F. Walther, and K. Hannig, Z Physiol Chem, 353: 95, 1972. referenced in (50).

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE